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SOME EFFECTS OF CARCINOGENS ON YEASTS

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With increasing knowledge of the chemistry of cholanthrene and related compounds and their action on mammalian cells, it has seemed desirable to study their effects on organisms with less complex structures and interrelations of parts. Yeasts were thought to be a group of plants of relatively simple organization and to lend themselves to comparatively simple techniques. A preliminary survey of a half dozen genera of increasing complexity of polarity and morphology showed much less effect on morphology than anticipated, therefore subsequent study was confined to two strains of *Saccharomyces ellipsoideus* Reess emend. Hansen, one a champagne strain, the other a Tokay strain. No differences in the physiology of these strains have been detected in our experiments.

We take this opportunity to acknowledge the generous financial assistance of the International Foundation for Cancer Research which has made this study possible; the careful work of Miss Helen Bramsch and of Stanley Hagen, who prepared sections of colonies for our studies of morphology in the early

stages of this work while research assistants; of Edward Cryder, who made most of the haemacytometer counts here reported and who prepared the graphs; and of Dr. Verne F. Goerger, who assisted in microphotography in our preliminary attempts to secure photographic data in connection with population studies. Mrs. Dodge has given much time during the four years of this investigation, while Dr. Johnson has been associated with us during the academic year 1939-40 only. We also wish to express our gratitude to Professors Philip Franklin and Norbert Wiener, of the Massachusetts Institute of Technology, for examining our graphs of population studies and for their assistance in interpreting them; and to Dr. Irving Langmuir and Dr. Katharine B. Blodgett, of the General Electric Company, for suggestions on our work with surface films.

MORPHOLOGY

An old stock culture of *Saccharomyces ellipsoideus,* which had been kept in the ice-box for nearly six months, produced on methyl cholanthrene agar (see p. 4) very curious colonies with a much more complex morphology than in the controls, or anything reported by Píšová ('34). These have been reported in a previous paper (Dodge and Dodge, '37). Repeated attempts to reproduce these curious colonies with their differentiated cortical and giant cells have so far failed.

Some differences in morphology exist between the cultures with a carcinogen, especially benzpyrene, and the controls in *Saccharomyces ellipsoideus* Reess emend. Hansen, *Zymonema capsulatus* Dodge, *Z. dermatitidis* (Gilchrist & Stokes) Dodge, *Mycocandida onychophila* (Poll. & Nann.) Lang. & Tal., and *Castellania tropicalis* (Cast.) Dodge (a strain originally from Castellani's laboratory but perhaps not the original strain), but these differences are much less striking and difficult to describe. That the age of the colony from which the transfer is made and its previous history have some effects on its growth is well known, but so far we have been unable to duplicate our first observations on *S. ellipsoideus* and have turned our attention to fermentation and growth studies.

Growth

Growth as applied to unicellular microorganisms is commonly restricted to multiplication or to increase in individuals, rather than to mere increase in size or differentiation and changes in tissues. Consequently, in any closed system such as a culture, it is essentially a measure of the increase in the number of cells between two instants of observation less the number that have died and disintegrated during the intervening time. In the absence of copulation and sporulation, multiplication is by binary fission, so that theoretically the number of cells should increase by powers of two, an ideal practically never reached in experimental conditions. Before discussing our results, it seems desirable to consider the methods which have been proposed and the sources of error and the interpretation of each method.

Methods and Sources of Error

Media.—In aiming to keep as many environmental and nutritional factors as constant as possible, the ideal medium would be a synthetic one where the concentration and interrelations of each nutrient were known. From time to time such media have been proposed for yeasts, but after trying various formulae, using both inorganic and organic (amino acid) sources of nitrogen, growth was so slow that we reluctantly returned to peptone in spite of its variable composition and the presence of possible growth-promoting substances. We have tried, however, to keep our sources of error as constant as possible by using only two batches of Difco Peptone in all our experiments and we have failed to find any significant difference between the two batches.

Our liquid medium has consisted of 1 per cent Difco Peptone and 2 per cent glucose. To this 2 per cent agar was added for solid media. Saturated aqueous solutions of the carcinogens[1]

[1] The carcinogen crystals were suspended in large flasks of hot distilled water and allowed to stand for several days with occasional shaking and the remaining crystals filtered off through fine filter-paper; the resulting filtrate was considered as a saturated aqueous solution.

were used to dissolve the other ingredients of the medium and distilled water for the controls. The medium has been autoclaved at 15 lb. pressure for 15 minutes. The 1,2,5,6 di-benz-anthracene, benz-pyrene, and methyl cholanthrene[2] have been used in the course of this study, using methyl cholanthrene for most of the experiments. The solubility of carcinogens is very slight, and so far we have been unable to secure a satisfactory method for determining it. We experimented with several monomolecular film methods developed or suggested by Langmuir and others without satisfactory results. The apparent monomolecular film resulting from spreading the methyl cholanthrene dissolved in benzene was evidently not a true monomolecular film, and Dr. D. F. Waugh, of the Department of Zoology of Washington University, after a further study of our material, reports as follows: Attempts to spread methyl cholanthrene, dissolved in benzene, resulted in the formation of a large number of extremely thin platelets which formed at the periphery of the benzene drop. The continuous formation of such platelets caused the water surface, for a considerable distance around the benzene drop, to be covered by a film of methyl cholanthrene which was apparently too thin to show interference colors but which, however, was a number of molecules thick. The effect was similar to that obtained when paraffin, dissolved in benzene, is allowed to spread. No film measurements were attempted.

In the solutions used, these carcinogens appeared to have a

[2] The 1,2,5,6 di-benz-anthracene was secured from the Eastman Kodak Company, the benz-pyrene from F. Hoffmann, Laroche & Co.A.G., Bâle, and the methyl cholanthrene from the International Foundation for Cancer Research. On checking the melting point of our methyl cholanthrene after it stood three years on a laboratory shelf, it was found to melt at 168–174° C. instead of 176–177° C. It was recrystallized by dissolving in hot benzene, cooling and precipitating with cold ether, when it melted at 176–177° C. It was also dissolved in benzene, picric acid dissolved in benzene added, heated with bone black, cooled, filtered, and dissolved in more benzene. Sodium carbonate was added, the flask shaken, filtered, the filtrate washed and recrystallized as above. This also melted at 176–177° C. A sample of methyl cholanthrene was also obtained from the Eastman Kodak Company but as it melted at 171–174° C. it was not used in our experiments. A careful inspection of our data, obtained shortly before and after our purification, revealed no significant differences. Alsopp ('40) has reported alteration of benz-pyrene and 1,2,5,6 di-benz-anthracene when irradiated with ultra violet light.

marked effect on surface tension. When the media were being made up in volumetric flasks and shaken well, the bubbles in the carcinogen media rose rapidly and broke almost instantaneously, while those in the control media persisted as froth for a long time. We have not yet had time to make quantitative measurements of surface tension.

Measurement.—Five methods of measuring growth of microorganisms have been frequently used: diameter of colony, volume, weight (wet or dry), nephelometry, and counting. These will be discussed in this order.

Diameter of Colony.—When an organism grows radially with equal rates in all directions, as is the case with many fungi, this method is rapid and satisfactory. It yielded some satisfactory results with *Zymonema* but was unsatisfactory for the moister colonies, as they were not of uniform height and contour, and was abandoned after preliminary studies.

Volume.—The culture is centrifuged in graduated tubes and the volume of the closely packed cells is recorded. Variations in speed and time of centrifuging, as well as shape of the cells, are possible sources of error. This method has the inherent disadvantage that the cells may be injured in the process, so it does not lend itself readily to following a population over an extended period of time. It also requires a considerable period of time or a relatively large volume of culture media to secure sufficient growth. Since it did not seem to have advantages over dry weight for our purposes, it was not tried.

Weight.—Wet weight, where the organisms are filtered and weighed immediately, has given reasonably consistent results to some investigators (Satava, '18) but seemed to have little except speed to recommend it as compared with dry weight. In some of our preliminary work we filtered by suction, using filter-paper in Gooch crucibles before turning to alundum crucibles. The coarser crucibles (R.A. 98 and R.A. 360) were suitable for filamentous forms, but R.A. 84, with an average pore size considerably smaller than the diameter of our yeast cells, were used in our work with *Saccharomyces ellipsoideus*.

We used Erlenmeyer flasks of 125-ml. capacity, containing 50 ml. of medium inoculated with 3 drops of a uniform suspension from a 5-ml. pipette, and incubated at 25° C. At the end of incubation, the contents were transferred to 50-ml. centrifuge tubes, using the usual precautions of chemical gravimetric technique. After centrifuging long enough to secure a clear supernatant liquid, most of the latter was decanted. Then the yeast, resuspended in the remaining liquid, was poured into the crucible and the tube thoroughly rinsed twice with distilled water. Filtration was by moderate suction (too great suction will crack the crucibles). The crucible was then placed in a drying oven at 70° C. Constant weight was usually reached in 8 hours, although 24 hours was allowed between weighings before the weight was accepted as constant. No attempt was made to read the balance closer than milligrams as it was felt that the next figure would be insignificant. After the final weighing, the crucibles were heated to redness to remove the yeast cells and were stored in a desiccator until we were ready to weigh and use them again. This method gave very consistent results, showing very little error in the technique.

This method is a measure of the total cells, living and dead, but does not include those disintegrated during the time interval. Its chief disadvantage lies in the relatively long time that must elapse between inoculation and the first data as it takes about two days to produce sufficient growth to weigh with much accuracy. As we shall see later, each culture behaves as a separate individual after inoculation, so that the three values of a triplicate set were seldom identical although the variation was not great. If we try to take samples from a larger volume of culture, sampling error is introduced as it is difficult to secure a uniform suspension for sampling and by the very process of sampling the equilibrium of the culture is upset. Also the dry weight varies somewhat with the depth and the area of the surface of medium exposed to the air and hence with the size of the flask and the volume of the medium. Consequently, dry-weight methods are ill adapted to a study of growth rates. A further source of error was introduced in our preliminary experiments as all but *Saccharomyces ellip-*

soideus of the species studied were pathogenic for man; consequently the cultures were sterilized before filtration to avoid accidental infection of the laboratory workers. Since considerable exosmosis is known to follow death, it is probable that exosmosis occurred during the autoclaving and subsequent cooling, probably roughly proportional to the time, a factor hard to control or evaluate accurately; hence our values of dry weight in the preliminary experiments are probably too low. This source of error was not present in our work with *S. ellipsoideus.*

Nephelometry.—This popular method was not used, as it was thought that the great variation in the size of cells and the presence of oil droplets and refractive granules would tend to render it unsuitable for our work. Subsequent studies by Loofbourow and Dwyer ('38) and Richards ('40) seem to support this conclusion.

Census.—Counting of microorganisms has long been the chief source of information of growth rates as changes in population may be observed at frequent intervals. This may be accomplished either by sampling a large culture at intervals or by direct observation of a small closed system under a microscope. In the former, the sample may be dispersed as an inoculum into fresh medium and the resulting colonies counted, a method commonly used with very small organisms such as bacteria which are not easily observed directly. It gives a measure of the total living cells in the volume of sample and, by implication, in the whole culture if sampling and dilution technique is adequate. It does not measure the dead and disintegrated cells which may have formed during the given time interval. Steinhaus and Birkeland ('39) have emphasized the importance of this disintegration in growth studies on *Serratia marcescens* Bizio.

As an alternative, the sample may be placed in a haemacytometer and counted directly over different areas, the counts being averaged and computed for the whole sample and, by implication, for the whole culture. This will give the viable and dead cells but not the disintegrated. Differential counts may

be obtained by staining the dead cells. We used this method while studying the possible cell types of our cultures and attempted to obtain differential counts. In general, the curves roughly parallel the counts in colonies under continuous observation, described in the next paragraph, in spite of the presumably greater oxygen tension of the medium. Both these techniques have inherent sampling errors and changes in equilibrium (much less than in dry-weight determination since the sample subtracts a much smaller volume from the culture).

Finally, we may use a haemacytometer as a micro-culture dish and keep the closed system under theoretically constant observation, counting the cells lying in the volume of medium above the ruled squares. In our work we used standard haemacytometers, taking a sufficient amount of culture medium to fill the counting cell without running into the grooves which were filled with vaseline. A suspension of yeast cells was made and diluted so that the volume used in the culture chamber would contain approximately 1 cell per small square when the cover-slip was lowered into place. No gas bubbles were produced under our experimental conditions. Care was taken to exclude all air bubbles as their expansion and contraction with relatively slight variations in temperature and barometric pressure might cause convection currents. A count was made immediately, and if the cells were found to be unevenly distributed or too many per small square, the culture was washed up and a new one started.

Counts were made at hourly intervals during the working day. It is evident that the observer will need time to sleep and rest his eyes so that a continuous record could not be secured with the observers available. The carcinogens were counted from 8:30 a. m. to 4:30 p. m., and the controls from 9 a. m. to 5 p. m. each day. When there were indications of a sudden change in the slope of the curve toward the end of the day we returned to the laboratory in the evening for additional counts. On Sundays only two or three counts each were made, unless the counts showed considerable change from the previous ones. In the graphs based on these observations, the daytime periods appear as sawteeth of varying

magnitude connected by straight lines. In this manner microcultures were observed more or less continuously up to 46 days. We probably missed many minor peaks and perhaps a few major ones, but our data were the best obtainable with the observers available.

Several difficulties seem to be inherent in this method. Oxygen tension must be lower than in flask cultures, in fact approaching anaerobic conditions. The volume of culture medium available per cell is probably less, although we have no data on this point. The error of counting is negligible until about 60 yeast cells per small square is reached when the cells are closely packed, and it increases as larger counts are obtained, tending to smooth the peaks of the curve more than the depressions. Another source of error lies in the movement of single cells, or rarely pairs of cells, over the line from one square to the next as the rows of small squares counted were not contiguous. While the yeasts are supposed to be nonmotile, several times we observed slow movements of translation similar to those common in some of the Myxophyceae, and a study of photographic records and circumstantial evidence point to other examples. The magnitude of the error is probably not great, as in general the movements would tend to cancel each other. It has been suggested that such movements are due to convection currents, but this seems unlikely since in all observed cases cells very close to the moving cell showed no movement. Such movements were noted on slides which had been resting on the stage of the microscope for 12–18 hours, as well as on those resting on the table beside the microscope. No attempt was made to use a constant-temperature stage, but in every case the slides lay side by side next to the microscope, covered by a small box cover to exclude dust and light when not under actual observation. Therefore, the temperature difference between the two halves of the same slide and between the two methyl cholanthrene cultures on one slide and the controls on the other were very slight, although there was some diurnal variation in the laboratory as well as some seasonal variation after the building was no longer heated, since these experiments covered a period from No-

vember 1938 to the middle of June 1939. These variations probably affected the total counts and consequently the growth rates slightly but less than other factors inherent in the experimental conditions.

The sampling error of the counts is probably low. It was not studied during the direct observation counts, but the photomicrographs showed it to be less than other sources of error in that method (see p. 19), since curves obtained by plotting counts from different rows of four small squares each are parallel for the same microculture. On the other hand, in a preliminary experiment where a small amount of air was included between the vaseline seal and the medium, due to a faulty seal, growth was more rapid (probably owing to greater oxygen tension) and some cells moved into the field, vitiating the experiments.

In our graphs, the points were plotted from the actual counts, and a factor of 50,000 should be used to express cells per cubic millimeter.

An automatic apparatus to produce photomicrographs at regular intervals suggests itself for reducing some of the errors of this method and giving a more continuous record than we have been able to secure by a limited number of observers. Some attempts were made to construct such an apparatus but it presented financial and technical difficulties in the conditions under which we worked. One inherent difficulty of this method is that many cells observed easily by focusing from the upper to the lower surfaces of the layer of liquid between slide and cover slip are missed by the focal plane of the camera, and the images of cells just below and just above the focal plane are so blurred that it is not easy to decide whether buds are attached to the cells or not. Therefore, one is forced to count only the cells in sharp focus. This increases the movement error much more than in direct observation, as well as the inherent difficulty of keeping a camera in the same focus over a period of several weeks while manipulating it to change spools of film, etc. It is probable that, with a specially constructed haemacytometer having a much thinner layer of

medium between slide and cover slip, a very bright light source of the flashing type, and a specially constructed camera, such a method might be devised but it was beyond our means.

Fermentation

Quantitative studies of fermentation fall into two main categories on the basis of determination of carbon dioxide evolved. The gas may be measured volumetrically in a gas burette, applying the usual corrections for temperature and pressure, and the weight computed. The 50 ml. of medium was pipetted into a 125-ml. Erlenmeyer flask, sterilized, and inoculated with three drops of a suspension of *Saccharomyces ellipsoideus*. The flask was attached to the gas burette by means of previously sterilized rubber stoppers and glass tubing. The gas was collected over acidulated water and read at regular intervals, recording temperature and barometric pressure from a mercury barometer hanging near the laboratory desk. After each reading the gas was allowed to escape and the water column returned to zero by means of a leveling bulb. Periods of a rapidly rising barometer complicated the process since they tended to drive the acidulated water back into the culture flask, causing the discontinuance of some experiments earlier than planned. This method has an advantage in allowing more frequent readings than gravimetric methods, with the same degree of accuracy.

In our later work we used gravimetric determination by collecting the gas in potash bulbs or soda-lime tubes and weighing. After eliminating several sources of error in preliminary experiments, we set up trains as follows: soda-lime tube, wash-bottle of distilled water, tube with sterile cotton, large test-tube with 50 ml. of medium, tube with sterile cotton, anhydrous calcium chloride tube, potash bulb or soda-lime tube which was weighed, closed filter flask (to prevent back seepage of water or carbon dioxide to the rest of the system), and water pump. Each time before weighing, the pump was started, and moist, carbon-dioxide-free air was drawn slowly through the system for ten minutes. The soda-lime tubes were weighed quickly

on a chainomatic balance. The tubes containing the medium were sterilized, inoculated, and rapidly attached to the system by previously sterilized rubber stoppers and glass tubing. No contaminations were detected. By the above system the air passing over the medium was moist, free of carbon dioxide and sterile, and the gas reaching the soda-lime tube to be weighed was dry, hence we were not weighing water vapor as well as carbon dioxide. A possible source of error is too rapid aeration, which might remove carbon dioxide from the soda-lime tube before it was completely absorbed. The final, practically constant, weights obtained in our longer experiments seem to support the accuracy of this method. Potash bulbs with strong solutions of sodium hydroxide were early abandoned, as water vapor is lost from the solution during the passage of air through it and in some of our early experiments the potash bulbs actually lost weight. While this method does not lend itself as readily to frequent readings, it is much simpler and involves less computation.

Besides measurement of carbon dioxide evolved, an attempt was made to study the amount of sugar disappearing during the growth of the culture. In our early experiments we determined sugar by the Benedict and Folin-Wu methods. Later we returned to the Bertrand-Shaffer method in which the Fehling's solution is reduced under standard conditions and the precipitate dissolved and titrated with standard potassium permanganate. The end-point of the titration is sharper by this method and gives more satisfactory results. Since other substances beside glucose reduce Fehling's solution, the interpretation of results is sometimes puzzling. In general, the reducing power of the medium disappeared sooner than the cessation of carbon dioxide evolution, although in the final computations the sum of the carbon dioxide, the remaining reducing substance (computed as glucose), and the dry weight (assuming it was all carbohydrate which it certainly was not) accounted for only half to three fourths of the original glucose (determined from the uninoculated tube). The reactions involved need further study before the data are satisfactory.

EXPERIMENTAL DATA

Colony diameter.—This proved a fair measure of growth for filamentous forms but less so for forms with a moist creamy colony. The methyl cholanthrene seems to have depressed

DIAMETER OF COLONIES IN MILLIMETERS

Conc. %	*Z. dermatitidis*			*C. tropicalis*				*M. onychophila* 10 days						*S. ellipsoideus* 20 days				
	10 days	20 days		10 days		20 days												
0	10	25	27	23	24	35		7	8.8	7	2	6		6	6	6.5	5	9
2	4	22	25	20	22	31					2	6					2	5.5
4	4	18	21	10	11	25	28				3	5					2	5
10	7	17	23	13	20	30		4	6	6	3	6		5	5	6	2	4
20	5	23	25	11	13	25	26				6	6	7				2	6
100	7	23	25	12	14	18	20	5	5	5	4	5	7	8	8	8	3	6

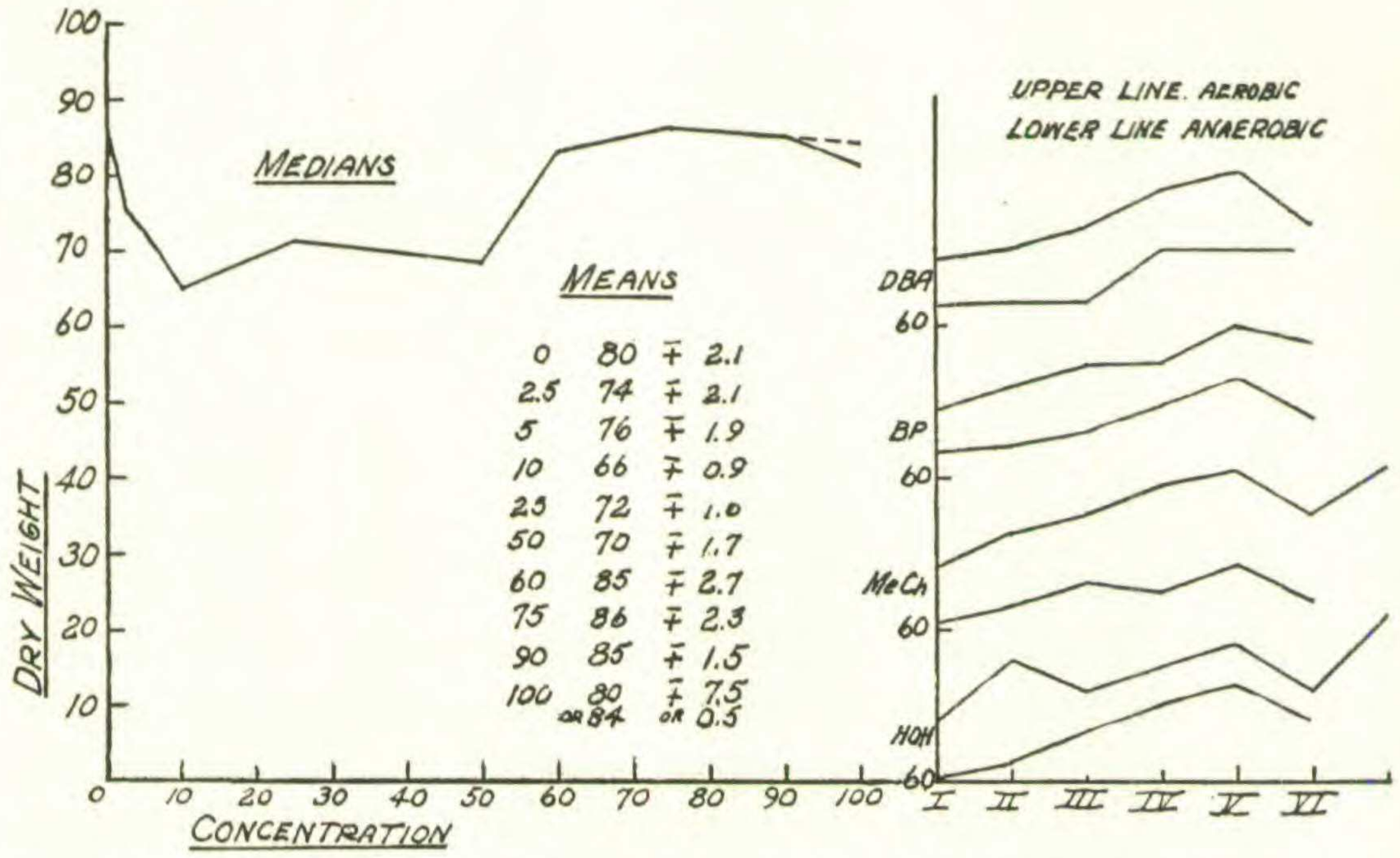
growth of *Castellania tropicalis* and perhaps of *Zymonema dermatitidis* in concentrations above 4 per cent saturation. Dry-weight determinations given below seem to bear this out although the figures are less striking. The variable thickness of the colonies of *Mycocandida onychophila* and *Saccharomyces ellipsoideus* results in seemingly erratic figures for these species.

Dry weight.—In our study of the influence of concentration of carcinogen on dry weight we secured the following results with *Castellania tropicalis* and *Mycocandida onychophila.* It should be remembered that these were our earliest experiments before our technique was perfected and that exosmosis following autoclaving may have affected the results.

DRY WEIGHTS IN MILLIGRAMS

Conc. %	*Castellania tropicalis*							*Mycocandida onychophila*									
	mg. dry weight					Average	Error	mg. dry weight								Average	Error
0	374	359	324			349	8.3	37			27	20		27	20	26.2	1.9
1			254	277	231	254	7.5			75	57	58	75	58	57	65.0	2.8
2	299		254	340	320	303	11.3	39		48	46	55	55	48	46	48.1	1.2
4								34	32								
5	316	315	276	284	318	301	6.6			46	54	23	54	46	23	39.0	3.3
10	285	285	316	321	328	312	6.3			38	21	27	38	27	21	28.7	2.1
20	340	326	307	332	309	323	4.5	93	105	10	61	27	61	27	10	49.1	8.2
50			329	347	335	337	3.1			11	30	8	30	11	8	16.3	3.1
100	191	221	279	339	330	272	19.8	59	69	52	14	29	52	29	14	37.2	5.4

In a subsequent series of experiments with *Saccharomyces ellipsoideus* we found the following results (Graph 1 left):



Graph 1. Left, growth in dry weight at varying concentrations of methyl cholanthrene. Medians plotted, means tabulated. Right, growth in dry weight with three carcinogens and controls. The upper line represents cultures under aerobic conditions, the lower line represents cultures used for fermentation experiments shown in Graph 3, where conditions were essentially anaerobic.

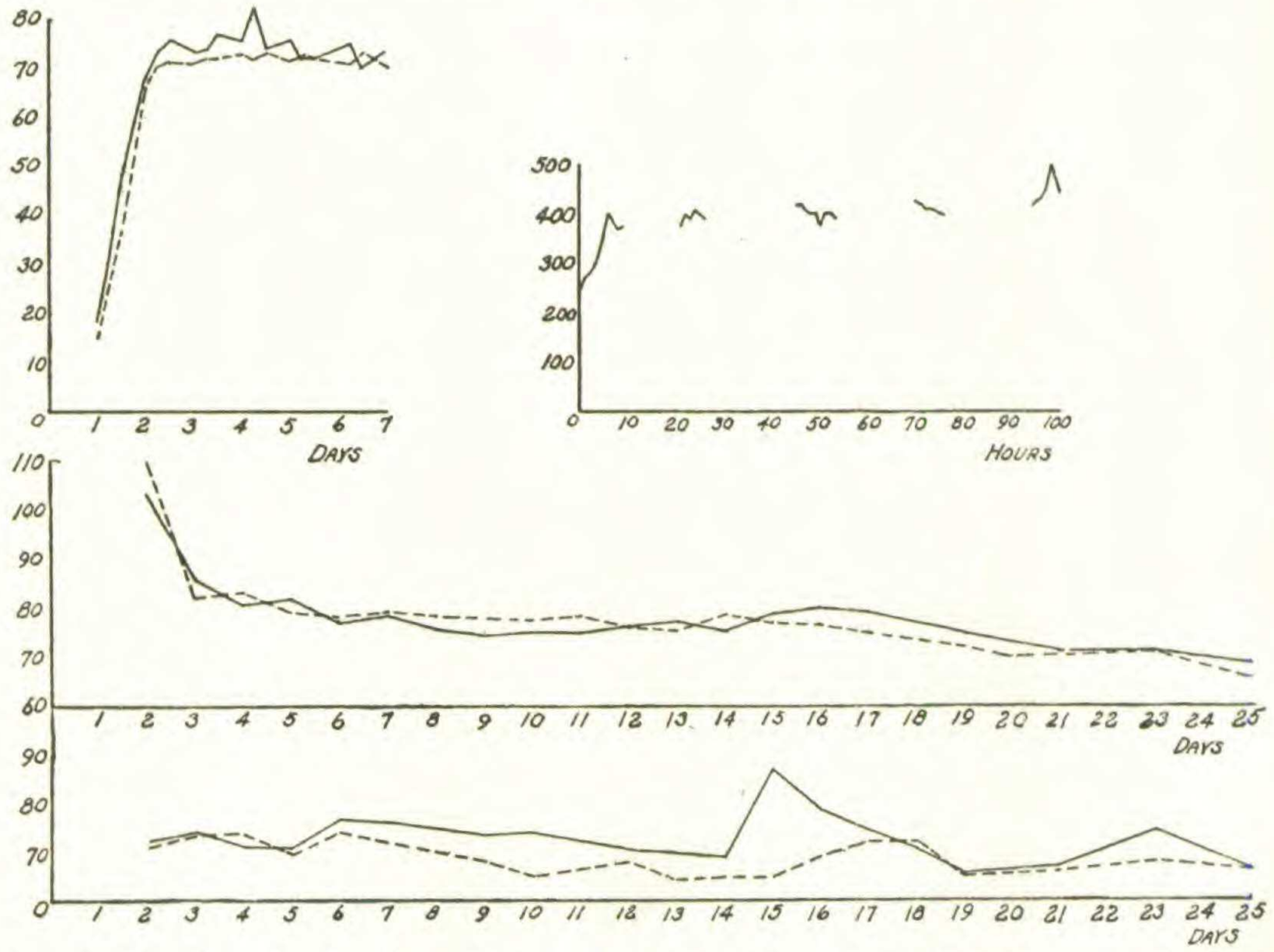
The above graph is based on 5 to 15 determinations for each concentration of the carcinogen (expressed in percentage saturation) with a probable error of ± 0.9–2.7 mg. While the results are not striking they seem to indicate that smaller concentrations depress growth as determined by dry weight over that of the controls, while concentrations over 50 per cent saturation tend to increase it to that of saturation. As the above averages include some of our earliest experiments before we had secured the alundum crucibles, they are probably less accurate than those of our later work. If we exclude these experiments the curves are substantially the same except the value for saturated methyl cholanthrene (shown by dotted line in the graph).

Effect of various carcinogens.—In connection with fermentation experiments, dry weights were determined in quadruplicate for methyl cholanthrene, benz-pyrene, and 1,2,5,6 di-benz-

anthracene and controls on cultures 7 days old. No significant differences were noted, as we obtained the following figures: control 70.8 ± 0.8 mg.; methyl cholanthrene 70.8 ± 0.8 mg.; benz-pyrene 70.8 ± 0.8 mg.; and 1,2,5,6 di-benz-anthracene 70.4 ± 0.8. In the light of our subsequent experiments, it seems likely that the time (7 days) chosen for our experiment was unfortunate, since the controls are the same as the carcinogen, probably due to crossing of curves. It is interesting to note, however, that if each separate dry weight is plotted, the successive weights for methyl cholanthrene are 64.5 ± 1.7, 68.0 ± 0.9, 71.5 ± 2.1, 74.2 ± 1.8, 77.2 ± 1.3 and 72.5 ± 1.5; 1,2,5,6 di-benz-anthracene is slightly aberrant with values 65.7 ± 0.8, 68.2 ± 1.3, 68.7 ± 2.2, 75.6 ± 2.0, 75.5 ± 2.2 and 71.7 ± 1.8 (Graph 1 right). This suggests some sort of cycle of dry weights such as we shall also see in fermentation. In these values the probable errors are higher owing to the averages being for 4 values instead of 24. The range of probable error for controls is ± 0.9–2.6; methyl cholanthrene ± 1.3–2.1; benz-pyrene ± 0.8–2.1; and 1,2,5,6 di-benz-anthracene ± 0.8–2.2 mg. In this series of experiments, half of the values were obtained in cultures freely exposed to the air as in our other dry-weight determinations. The other half were used to determine fermentations by gas volumes which in the conditions of our experiments were under lower oxygen tension and high carbon-dioxide tension approaching anaerobic conditions. In the latter the absolute amounts were about 10 per cent less dry weight and more variable in successive experiments. The probable error of the determinations ranged from 0 (when two observed values were identical) to ± 1.2 mg.

Several long-time experiments were undertaken to attempt to follow changes in dry weight during the stationary and decline phases of cultures. Dry-weight determinations do not lend themselves readily to a study of the lag and the logarithmic phases. The differences between methyl cholanthrene cultures and controls are slight. As the curve levels off, it begins to descend slowly by a series of small sawteeth, similar to those of population counts but much less in extent, with an occasional high peak. While in general the curve for methyl

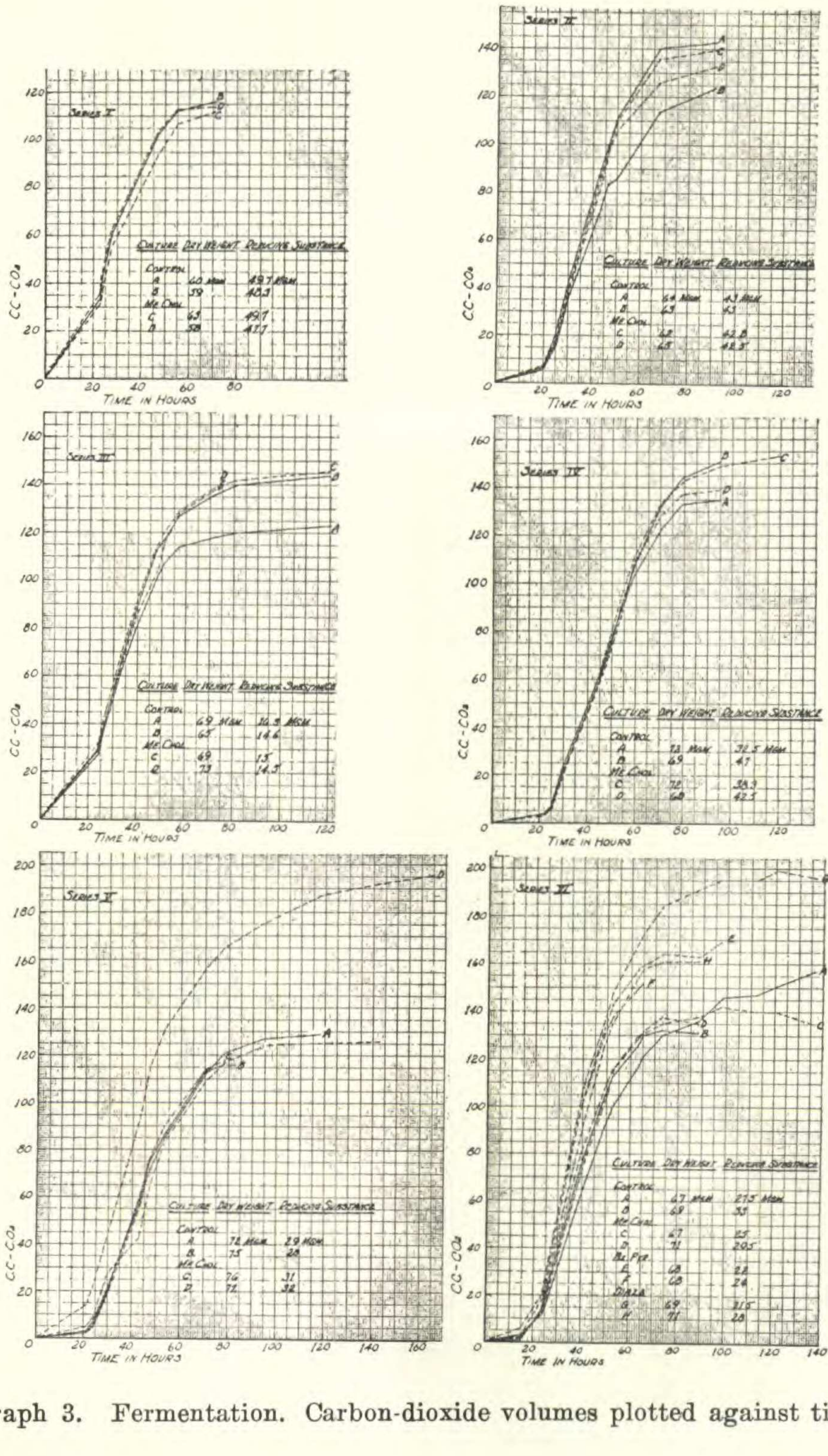
cholanthrene is slightly below that of the control, the curves frequently cross. An average of 118 determinations from the second to the twenty-fifth day gives a dry weight of 80.1 for the controls and 78.1 for the methyl cholanthrene cultures, with a probable error much less than ± 1 mg. While only a single experiment was carried to 130 days, the results are similar to the above series carried only to 25 days (Graph 2).



Graph 2. Growth in dry weights for first 25 days in long-term experiments. Upper right, a typical population curve with large inoculum. Note nearly complete absence of lag phase.

FERMENTATION

In our first experiments we attempted to study the effects of methyl cholanthrene, benz-pyrene and 1,2,5,6 di-benz-anthracene. Six successive series were run and the volumes of carbon dioxide recorded. When the corrected volumes are plotted against time, in series I–IV (Graph 3) we note a continual increase in the amount of gas evolved, the close proximity of the curves of carcinogen and control, and the rising dry weights in each successive series. In series V the total carbon dioxide is below that of series IV in the control and methyl cholanthrene, while that of 1,2,5,6 di-benz-anthracene is far ahead.



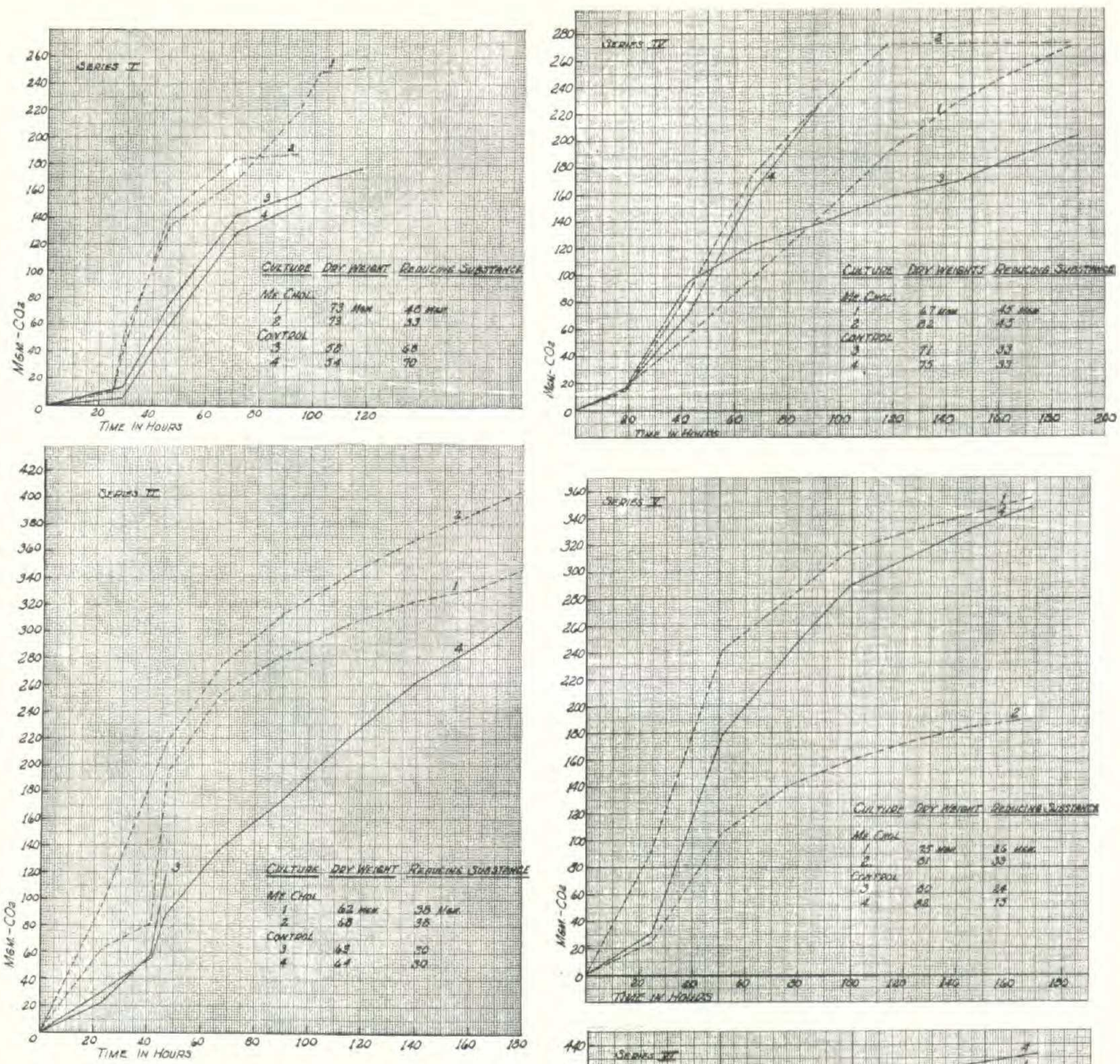
Graph 3. Fermentation. Carbon-dioxide volumes plotted against time.

In Series VI the control curve becomes irregular and both benz-pyrene and 1,2,5,6 di-benz-anthracene show great increases over series V. From our later experience, the irregularity of the control curve suggests that had we continued a seventh series we would have found the yeast much more susceptible to the influence of the carcinogens.

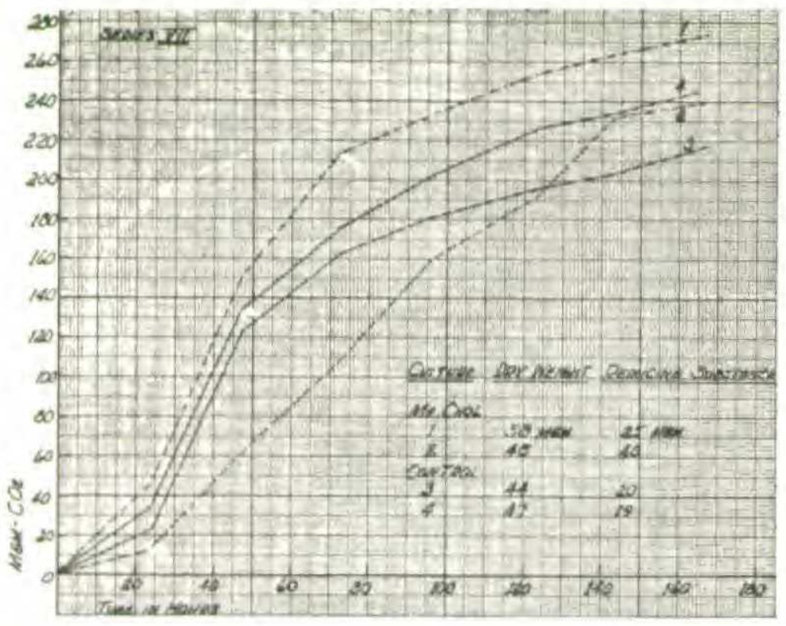
An attempt was made to determine the reducing power of the solutions at the end of the experiments, hoping to check the carbon dioxide evolved against the decrease in glucose. The percentages for the different series were as follows for the controls: I, 38.1; II, 40; III, 41.5; IV, 49; V, 48; VI, 45. Therefore it seems probable that part of the glucose is synthesized into a more complex carbohydrate (without or with part of the peptone) which remains in solution but which has lost its reducing power.

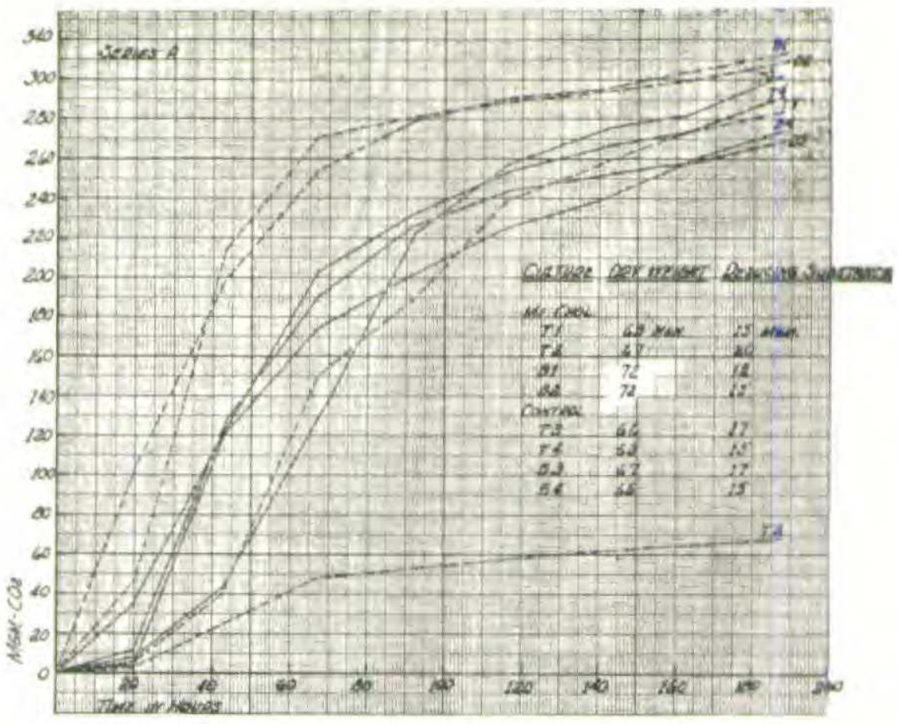
Since difficulties were experienced with negative pressures toward the close of the experiments, we turned to gravimetric determinations during the next experiments and confined our attention to methyl cholanthrene (Graphs 4, 5 upper left). The weights of carbon dioxide were higher in seven consecutive series and consequently the proportion of the original glucose accounted for was higher, ranging from 27 to 55 per cent for controls and 29 to 57 per cent for methyl cholanthrene, yet there was no conspicuous correlation between dry weights, weight of carbon dioxide, and the amount of glucose left. Since from other observations we had noted some differences in size and shape of cells, it seemed possible that the different types of cells might behave differently, and we attempted a partial separation by their different sedimentation rates (Satava, '18, further studied by Nielsen, '37).

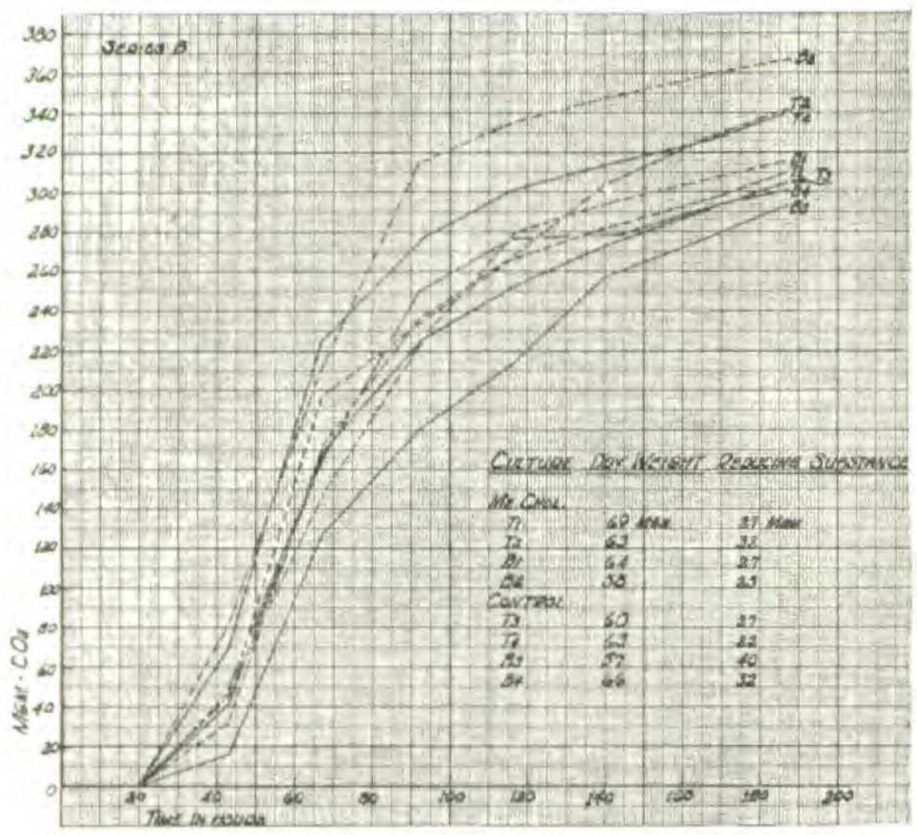
A 50-ml. culture to be used for inoculum was centrifuged until the cells, packed in the bottom, differed from those remaining in suspension, as shown in haemacytometer counts. One of the samples was then diluted until haemacytometer counts showed approximately the same number of cells per unit volume. These were used as an inoculum for Series A–G, top; while the sediment was used for another series (A–G bottom, Graphs 5, 6 left). A similar separation was also

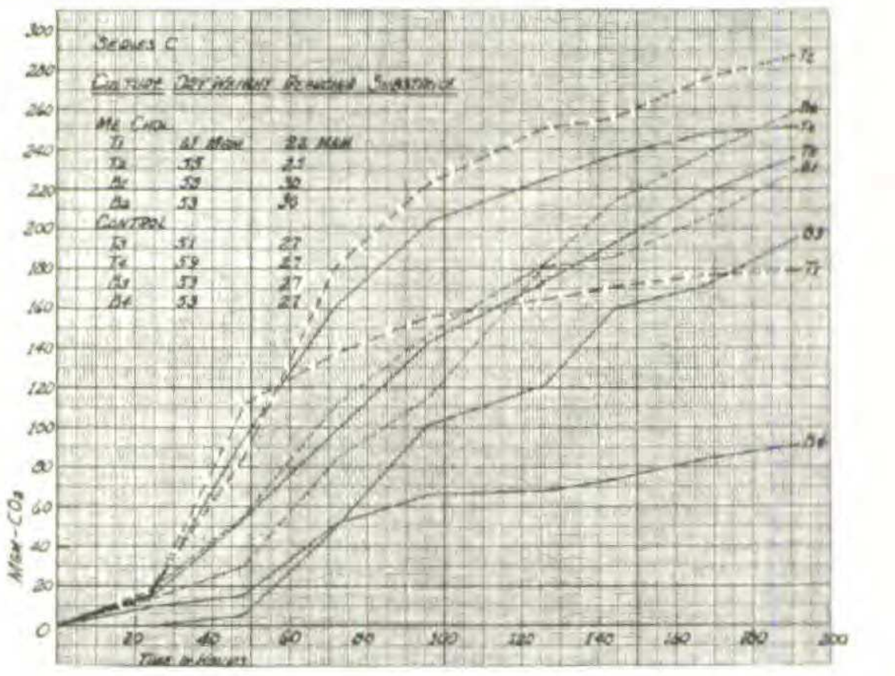


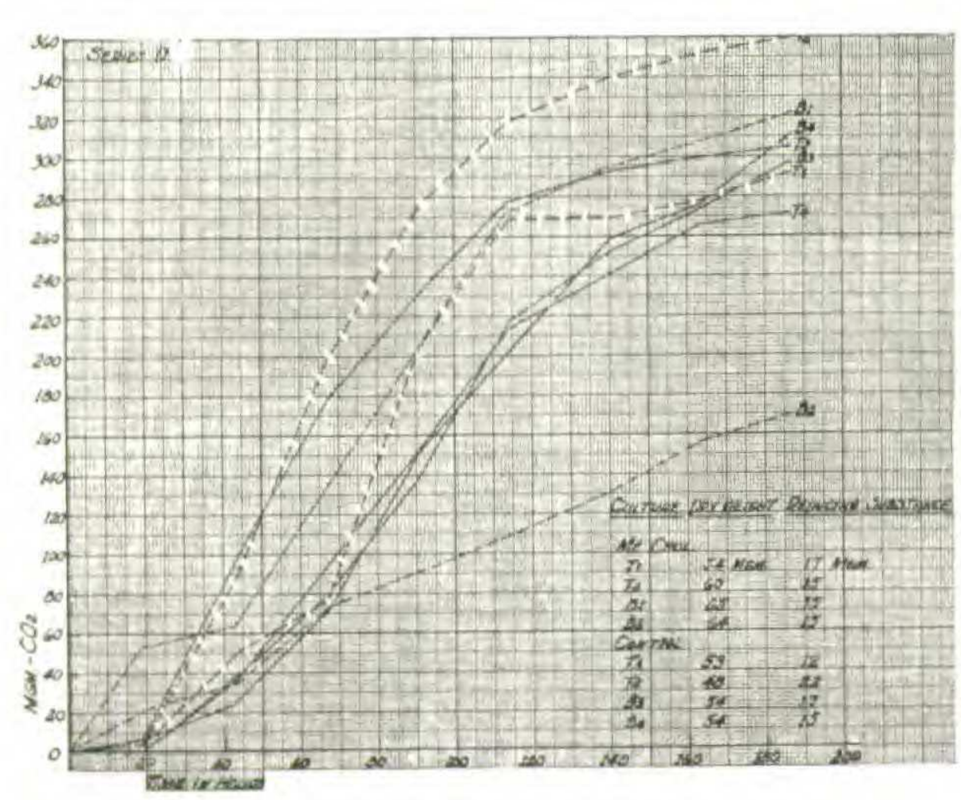
Graph 4. Fermentation. Another series with weights of carbon dioxide plotted against time. The last member of this series shown on upper left of Graph 5.

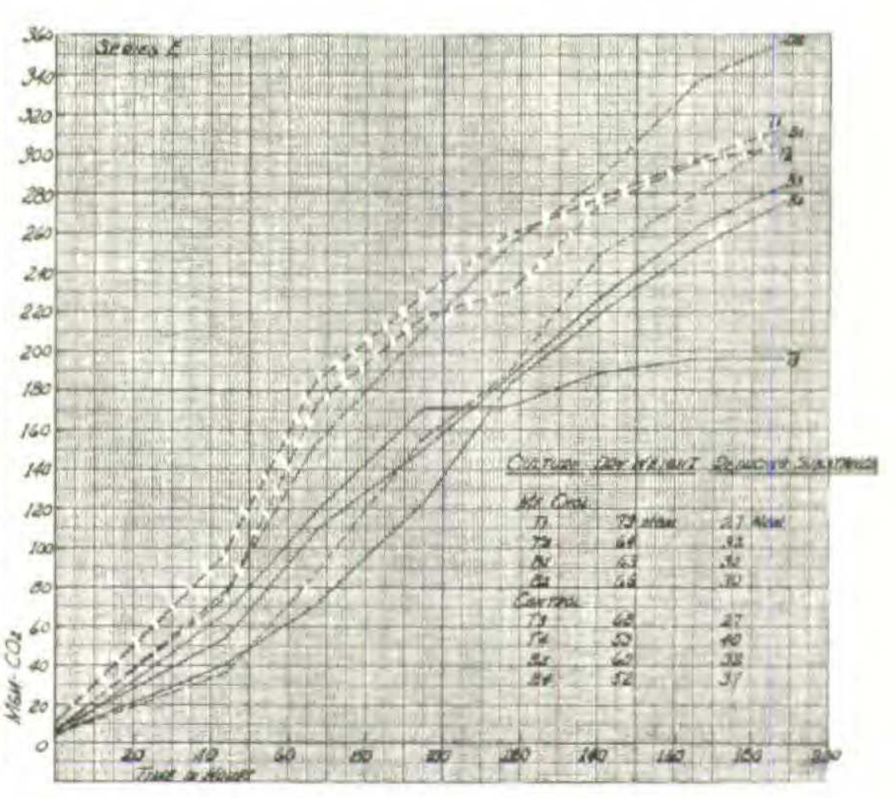












Graph 5. Fermentation. Upper left, continuation of series on Graph 4. Series A-E, contrast of fermentation by cells from the supernatant (T) and sediment (B) in fractional centrifugation studies.

attempted by natural selection with Gorodkova solution cultures and compared with our usual medium (Series H, Graph 6 upper right) and finally a comparison of the top (T_1, T_3) and bottom (B_2, B_4) cells with the whole (S_1, S_2, S_3, S_4) culture (Series I, Graph 6 lower right). For discussion of the morphological types found, see p. 25).

With both the top and bottom cells the controls show less dry weight and leave more glucose in solution at the end of the experiment than in the methyl cholanthrene. The top cells of the controls show more of the glucose accounted for and slightly greater total fermentation than the methyl cholanthrene cultures, while the bottom cells of the controls show less glucose accounted for and slightly less fermentation. Since the fermentation was allowed to go to completion, it would be expected that the difference in total fermentation would not be conspicuous. If we examine the amount at about 90 hours, when the curve begins to level off, the differences are more conspicuous, especially in the bottom methyl cholanthrene. As we shall see later, there was no conspicuous correlation between these data and the various types of cells present. It seems probable that such relations exist but it will take more refined methods of classifying the morphology of cell types to show it clearly.

Population Studies

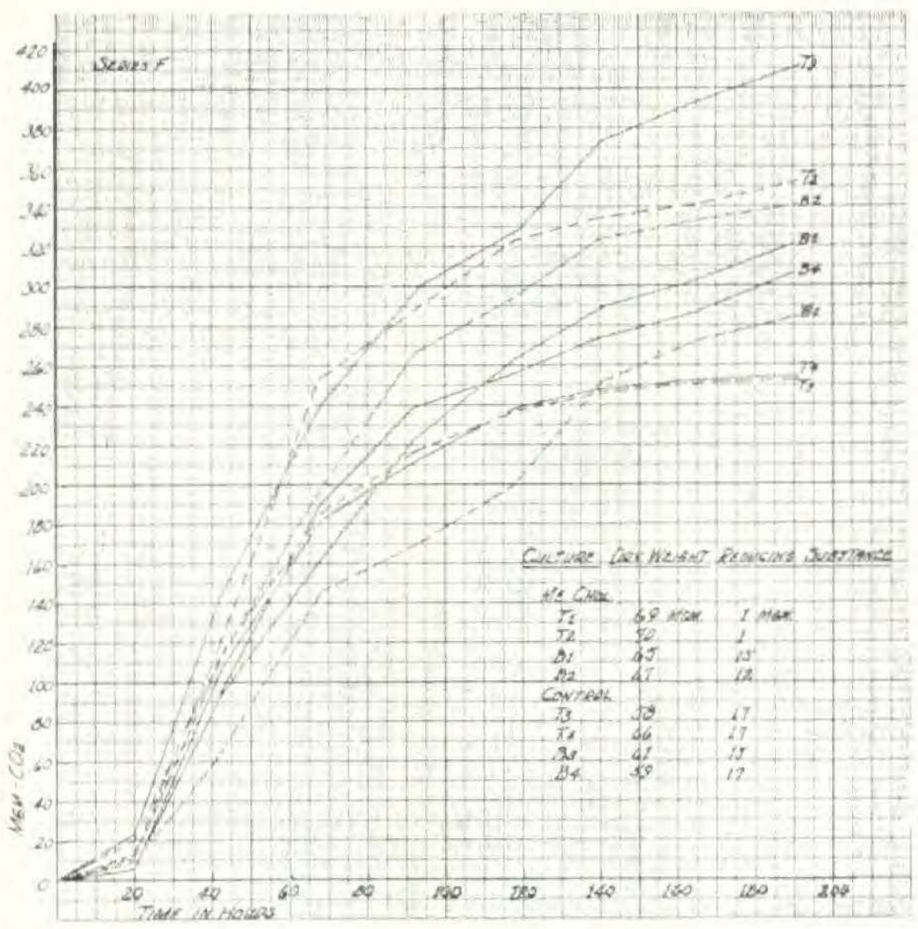
In order to test the validity of our population counts, a given large square of the haemacytometer was photographed at 4–6-hour intervals for 150 hours and the resulting negatives were used to count the cells in each small square. Graph 7 left was based on the sum of rows 1 and 3 and of rows 2 and 4 to compare with our previous counts where we counted rows 1 and 3 in each of two adjacent large squares.

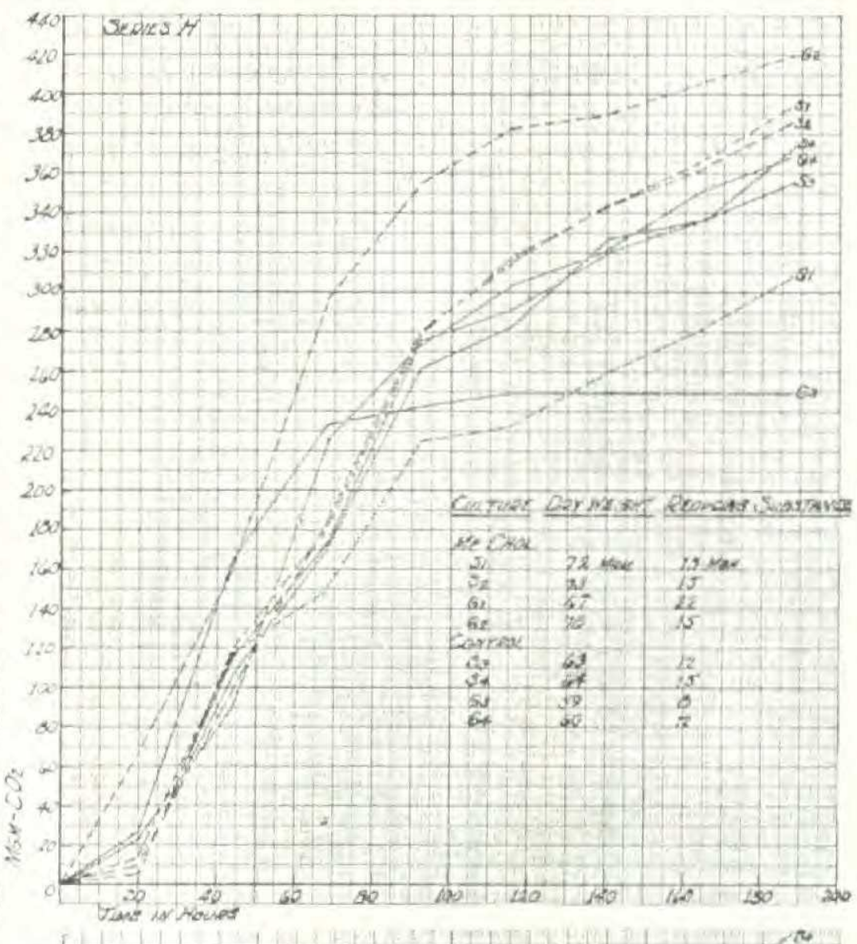
The curves are closely parallel considering the difficulty of keeping the microscope at the same focus over a long period of time. This fact should rule out serious error due to the movement of cells from one square to another, and points to a single stimulus or group of interrelated stimuli which act on the whole population within the time intervals between photographs. As

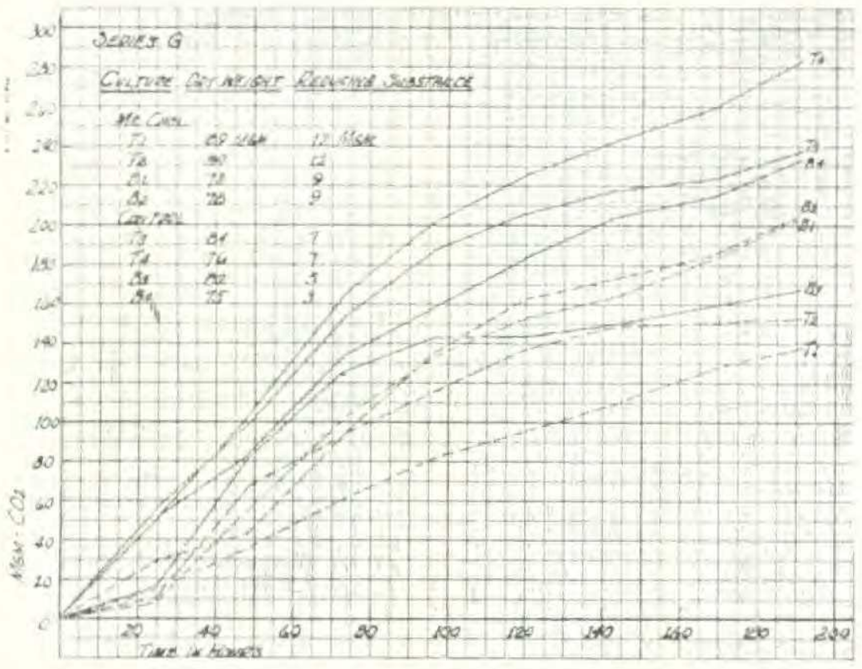
a further check upon our method we plotted, from a single field of our haemacytometer, the hourly counts selected at random from our mass of data and again found the curves parallel. In this case rows 1 and 3 in two adjacent large squares were counted. If we plot the average of rows 1 and 3 from each large square the parallelism is closer than if we compare the two rows farther apart, i.e., row 1 of the upper large square with row 4 of the lower large square (Graph 7 lower right), suggesting a stimulus diffusing from a focus which takes more than an hour to reach its maximum effect. Since these curves are extremely irregular with similar phase throughout the community, it is clear that there is some factor or group of interrelated factors acting uniformly upon the whole community.

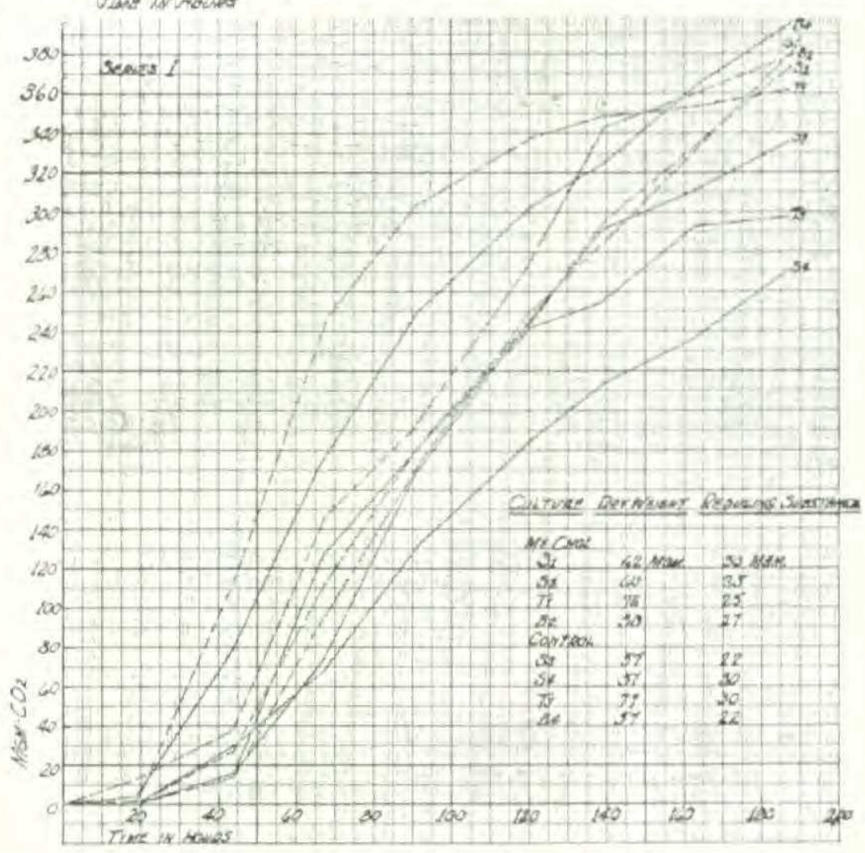
In the course of our experiments 36 cultures were counted for varying periods of time. If we average all our counts where there were at least 10 data available at each hourly interval, we have curves approximating the classical growth curve, since most of the fluctuations are averaged out (Graph 7 upper right). The control curve shows a lag of 12–15 hours, a logarithmic phase of about 30 hours and a decline phase of at least 60 hours, followed by a smaller secondary rise of at least 60 hours. The slope of the secondary rise is less steep and the total counts are considerably less. Richards ('32, '40) has studied this secondary rise in much detail, using *Saccharomyces cerevisiae*. Our data parallel that of Richards rather closely considering the differences of medium and species of yeast.

Similarly, the average curve of the carcinogen is smooth, showing a lag of 20–22 hours, a logarithmic phase of about 80 hours, a short decline of about 20 hours, and secondary and tertiary rises of about 25 hours with declines of about the same length. The rises are less steep and the height of the first peak is less, but the decline phase tends to remain at a much higher level than in the control. Fishbein, Weaver and Scherago, in a paper read at the annual meeting of the Society of American Bacteriologists as this paper was in press, working with *Escherichia coli* and 1,2,5,6 di-benz-anthracene, showed essen-

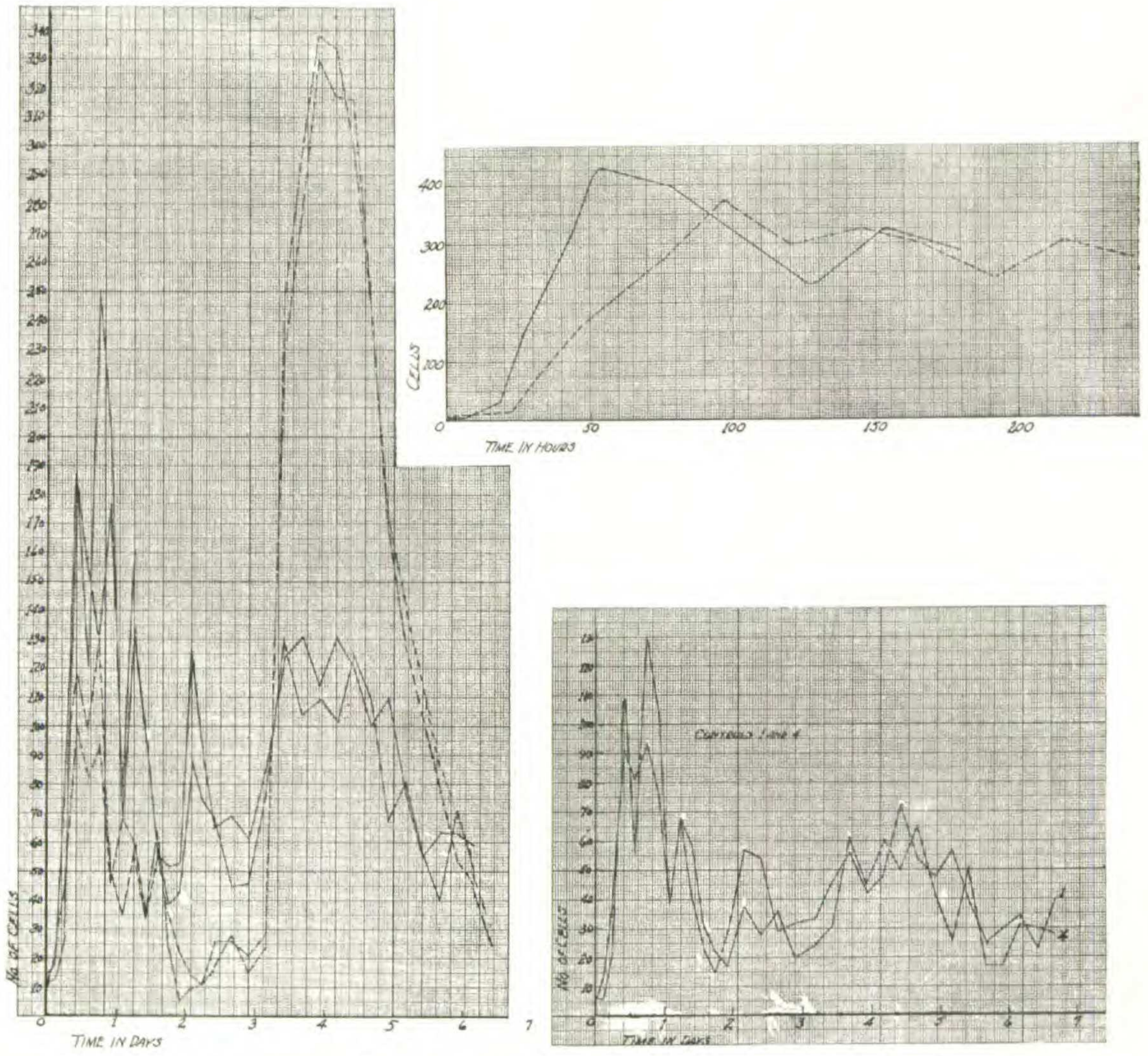








Graph 6. Fermentation. Left (Series F, G), continuation of series on Graph 5. Series H, comparison of our standard medium with inoculum from Gorodkova solution with its higher percentage of elongate cells. Series I, comparison of fermentation by uncentrifuged inoculum with the supernatant (T) and sediment (B) from fractional centrifugation.



Graph 7. Lower graphs, cell counts from photographs at 4–6-hour intervals. Left, sums of rows 1 and 3 plotted against rows 2 and 4 as in Graph 8 of haemacytometer counts. Note parallelism of curves. Right, controls only, row 1 plotted against row 4. Upper right, average curve for all curves shown on Graph 8.

tially the same type of curves, although working with bacteria and colloidal suspensions of the carcinogen.

In three cases where we started with higher inocula (about 200 cells per field, Graph 8 upper and extreme right, Graph 2 upper right) the curves rise, with no appreciable lag, to a much lower peak and level off for the first 100 hours in a long stationary phase (senescent phase of Steinhaus and Birkeland). This curve somewhat resembles that reported by Hopper and Clapp ('39) and closely that of Fishbein, Weaver and Scherago, mentioned above, when they used large inocula. These results agree essentially with those found in dry weights and fermentations where also we were dealing with large inocula. There is no appreciable difference in the slope of the curves between cultures grown in the haemacytometer and those grown in test-tubes and sampled for counting.

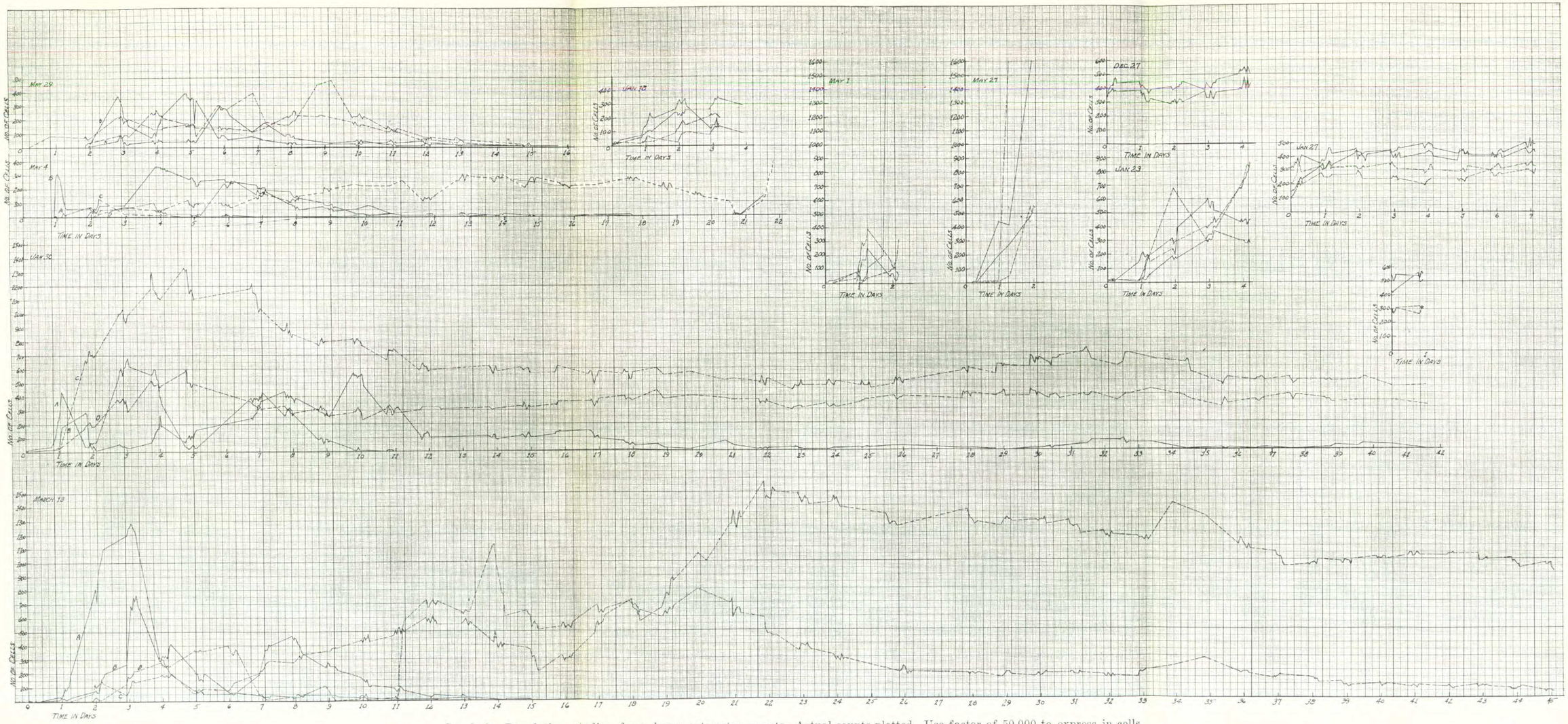
Averages, however, often conceal some important data. If we examine the curves of cultures in the two fields of the haemacytometer having the same medium and inoculated with approximately the same number of cells from the same suspension and subjected to the same environmental conditions but not physically connected, we find that they rapidly get out of phase and vary quite independently. If the fluctuations of the counts were due to genetic or to other factors inherent in the inoculum or factors of the external environment of the culture, we should expect the fluctuations to occur simultaneously in the separate cultures. This is not the case, and we may safely conclude that the fluctuations in the populations, resulting from division and disintegration of cells, are not the result of inherent variations in the inoculum nor in the external environment of the culture but rather the result of stimuli arising within the culture itself. Steinhaus and Birkeland ('39) probably observed similar fluctuations in *Serratia marcescens* Bizio but they present only average curves. They also emphasize the importance of disintegration of cells and the reutilization of the nutrients thus freed during the senescent phase.

Our curves (Graph 8) show a high degree of randomness with large fluctuations compared to the mean, such as one encounters in curves in stock-market reports where the deci-

sions of relatively few individuals in the community profoundly affect the curve of prices of stocks. Applying the usual tests, we find that our curves are neither those of random distribution nor of Brownian movement. We should hardly expect random distribution since each new cell originates from a previous cell at a definite point in time and space, and from our fragmentary observations when light conditions were especially favorable, it seems to be included in a thin gelified sheath for some time after it has severed observable protoplasmic connection with the parent cell. While movement of single cells has been noted, presumably after the sheath has disintegrated, it has none of the characteristics of Brownian movement.

If the phenomena causing the fluctuations were mass effect due to large numbers of cells, we should expect large smooth sweeps with a small degree of randomness. In a large culture such as we used in determining dry weights and fermentation, we find this type of curve, since the small individual fluctuations arising at different points in time and space rapidly average out and give a relatively smooth curve with only the extremely large fluctuations evident. We have already seen this when we averaged all our individual curves where at least 10 counts for a given hour were available (Graph 7).

In the light of our experiments, it seems probable that we are not dealing with individuals in a community, but that the community as a whole represents a liquid tissue in which the cells have lost their geometric arrangement in space but have still retained some differentiation in function, and that certain cells are differentiated to secrete some growth hormone. Perhaps this unlooked-for result should have been expected, since from studies of comparative morphology and phylogeny of the yeasts we have a definite degeneration series from organisms with very complex morphology and life cycles to the "unicellular" condition in *Saccharomyces* (Dodge, '35). The presence and persistence of the gelified sheath point in this direction but need much more study before their meanings are clear. We also need more continuous observation by some method which would give a continuous record of the fluctuation in number of cells, paralleled by frequent microscopic examina-



Graph 8. Population studies from haemacytometer counts. Actual counts plotted. Use factor of 50,000 to express in cells per cubic millimeter. Graph of January 30, a single experiment with benz-pyrene; all the others with methyl cholanthrene. Broken lines, curves of carcinogens; solid lines, controls.

tions to locate the few individuals which are producing the fluctuation and to describe their morphology.

If we examine the individual fluctuations, we also note that very rapid "birth" rates are followed by nearly as rapid disintegration rates, so that the curve for a single fluctuation is nearly symmetrical. The very rapid disintegration of cells from one hour to the next has been evident in most of our work and seems to have been overlooked by many workers. This rapid disintegration apparently sets free nutrients which may be used again to allow more growth, for if we examine the bottoms of the depressions we find that the curves are again approximately symmetrical, although the peak reached after a depression is usually lower than the previous peak.

When we examine the curves for methyl cholanthrene we find essentially similar conditions. In general, the initial lag period is longer and the peaks are broader as well as higher. It would appear that the stimulus is more continuously applied in addition to that of the postulated hormone secreted by individual cells. The larger numbers of cells usually present after the first rise would also tend to smooth the curves, as there would be a greater chance of "hormone"-secreting cells being present. If the methyl cholanthrene is a constantly acting stimulus, it would tend to mask the rapid disintegration rates, thus resulting in increased growth rates during the logarithmic phase and apparently decreased disintegration rates. Since this does not appear to be the case, it is probable that the methyl cholanthrene stimulates cells to divide that ordinarily would not do so, apparently injured or senescent cells.

If a diffusible chemical impulse were the only factor, and all cells receiving the stimulation divided immediately, the peaks would be much greater and would reach approximately the same heights where the initial cultures contained approximately the same number of cells. Hence it seems probable that at any one time only a portion of the cells are sensitive to stimulation. If such cell sensitivity were merely a function of age, the curves would rise in continuing peaks as all the cells came of suitable age, which is not the case. The differences in height, number, and time of appearance of the peaks suggest rather

a few sensitive cells which form centers of growth. The height may be determined by the number of sensitive cells and whether such cells are in our immediate field of vision. Their age may be important in determining the response to the stimulus. Where there are very few sensitive cells, as in a small inoculum, the peak is conspicuous and sharp; where more of these cells are present, the peaks appear to be made up of a succession of sawteeth, less sharp as more sensitive cells are present until in large inocula we reach a broad and smooth curve similar to the classical growth curve. The other cells, which are present in far greater numbers than the sensitive cells, may be regarded as somatic cells. It is not clear at present whether they die slowly or persist until their function is taken over by a new crop of somatic cells.

That these cells are merely reproductive cells which function without a definite stimulus is untenable, since if there were no diffusible stimulus there would be no parallelism between curves plotted from different regions of the same culture. We would have only isolated centers where multiplication and disintegration would start quite independently, which is not the case.

There is also an effect traceable to the history of the culture evident through a series of transfers, since otherwise there would not be as much parallelism between subcultures transferred on the same day. Despite differences great enough to indicate that they vary independently, there is sufficient parallelism in population counts, and even more in dry weights and fermentation data, that duplicates, i.e., contemporaneous subcultures, are definitely more alike than cultures from the same stock culture made days or weeks apart.

There is still another complicating factor. Evidence is accumulating that there is a definite cycle of fluctuations in the physiology of the cultures. This cycle appears to be a long one, and while frequent transfer may somewhat modify the time it does not alter the fundamental process. It appears that instead of being a strictly unicellular organism, completing its life history in a comparatively short time, the yeast is a complex organism in which the whole culture behaves as an indi-

vidual, a liquid tissue with a much longer life history which the addition of fresh nutrient (transfer, which is similar to tissue cultures and cuttings of the higher plants) may alter only in a minor way. Taking long enough periods we may reproduce dry weights and fermentations very closely. Reappearance of very high dry weights (over 90 mg. under our standard conditions) in approximately six-month periods suggests this as an approximation for the cycle of our stock culture.

Cell Differentiation

Study of cells of individual cultures reveals a fairly constant morphology during the logarithmic phase. The inoculum, if taken from an actively growing 24-hour culture, consists of comparatively small, homogeneous cells. If inoculated from older cultures this form is also assumed during the lag phase and remains homogeneous during a portion of the logarithmic phase. Small vacuoles appear and granules of reserve food become visible in the cytoplasm. In still older cultures, the vacuoles become large and the granules more numerous, there being apparently little correlation as to whether the granules are located inside or outside of the vacuole itself. Elongate cells rarely occur in young cultures but are relatively more abundant as the culture ages, although their number is seldom large. In our medium, the elongate cells seem to revert easily to the spherical form but those isolated by a micromanipulator and transferred to fresh medium frequently retain the elongate form for several generations. Although cultures of the same age are relatively constant in morphology during the early stages after transfer, the morphology is progressively more variable in older cultures, perhaps due to different disintegration rates.

In this preliminary study we recorded the cell types as percentages of large spheres, small spheres, and elongate cells. We also recorded the spherical cells with buds, the elongate cells with buds, cells with large vacuoles with or without reserve materials, and shadow cells. In our experiments with fractional centrifugation we recorded the following: